The Metabolic Status of Diapause Embryos of *Artemia franciscana* (SFB)

James S. Clegg[1,*]
Laurie E. Drinkwater[2]
Patrick Sorgeloos[3]
[1]Bodega Marine Laboratory, University of California, Davis, Bodega Bay, California 94923; [2]Rodale Institute Research Center, Kutztown, Pennsylvania 19530; [3]Laboratory of Aquaculture and *Artemia* Reference Center, University of Ghent, Rozier 44, B-9000 Gent, Belgium

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Abstract

The brine shrimp Artemia franciscana *is widely used in aquaculture and basic research in areas ranging from molecular biology to evolution and ecology. A key feature of its life history involves the production of encysted embryos whose development is halted (enters diapause) at the gastrula stage. These shelled embryos are released into the aqueous environment where diapause continues until terminated by suitable conditions that produce an "activated embryo," which then can resume development when conditions permit. Very little is known about the metabolism of diapause embryos, in contrast to activated embryos, which have been studied extensively. We have examined selected features of metabolism in diapause embryos produced in laboratory cultures and collected from the field. Although in a state of developmental arrest, newly released diapause embryos are shown to carry on a vigorous metabolism. However, as diapause continues metabolism slows until its detection becomes an experimental problem; it is possible that metabolism comes to a reversible standstill. We also present results from studies on diapause termination and the resumption of metabolism. Finally, we will show that a major protein (p26), previously implicated as a potential molecular chaperone in activated embryos undergoing anoxia and thermal shock, behaves similarly in diapause embryos. Although direct evidence is lacking, that result suggests a role for p26 that may be common to the mechanisms involved with the control of diapause as well as the stress response in this system.*

Introduction

Diapause, a state of obligate dormancy, is widespread in nature, providing a way to resist and survive adverse conditions and/or to synchronize appro-

*To whom correspondence should be addressed; E-mail: jsclegg@ucdavis.edu.

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priate life cycle stages with suitable environments (see Lees 1955; Clutter 1978; Yamashita and Hasegawa 1985; McNamara 1994). In species of the brine shrimp, *Artemia,* diapause occurs in gastrula embryos that develop within the maternal female, are encased in a complex shell, and are then released to the surrounding aqueous environment (reviewed by Lavens and Sorgeloos [1987] and Drinkwater and Clegg [1991]).

The biology of *Artemia* spp. is well known and the subject of several reviews and books (Sorgeloos et al. 1987; MacRae, Bagshaw, and Warner 1989; Warner, MacRae, and Bagshaw 1989; Browne, Sorgeloos, and Trotman 1991). This literature documents the vital role that the diapause embryo of *Artemia* plays in nature, often being the only surviving stage of the life cycle. While some information is available on the environmental conditions that induce and terminate diapause (Lavens and Sorgeloos 1987; Drinkwater and Clegg 1991), very little is known about the metabolism of diapause embryos. To our knowledge only three papers have been published on this aspect (Clegg 1965; Drinkwater and Crowe 1987; Van der Linden et al. 1988), and these contain scanty metabolic information. A major cause of this shortage is the difficulty of obtaining diapause embryos in amounts that are adequate for biochemical research, a circumstance that contrasts with activated (dried) embryos, available commercially in virtually unlimited amounts.

In the present article we will show, among other things, that the newly released diapause embryo is indeed metabolically very active but that this metabolism is markedly reduced as diapause continues.

Material and Methods

Sources of Diapause Embryos

Artemia franciscana from the San Francisco Bay area (SFB) were purchased from San Francisco Bay Brand, Hayward, California, in 1978 as dried (activated) embryos, often referred to as "cysts." These were used as controls and also to produce adults in laboratory cultures from which diapause embryos were obtained. Seawater (SW) was reduced by evaporation to 75% of its original volume, and the inoculated *Artemia* larvae were fed on the microalga *Isochrysis* sp. and suspensions of baker's yeast. After sexually mature adults appeared in these cultures, filter paper strips were placed at the liquid-air interface to which the floating diapause embryos adhere and were collected. The studies shown in table 1 used these embryos.

For the respiration studies shown in figure 1, embryos were collected from the salterns in south San Francisco Bay and returned to the laboratory.

The company that harvests *Artemia* cysts from these salterns (San Francisco Bay Brand) collects them once a day during the harvesting season, removing the vast majority. Because we made our collection during one of these harvests (May 1989), the vast majority of the embryos used in this part of the study (fig. 1) were released from females over a 24-h period. After thorough washing and a brief sodium hypochlorite treatment (2.5% for 2 min at 4°C) the embryos were stored in the dark in aerated 0.25 M NaCl containing streptomycin (50 $\mu g \cdot mL^{-1}$) and penicillin (50 $IU \cdot mL^{-1}$) until respiration measurements were performed.

Since 1985, the *A. franciscana* (SFB) strain has been used for cyst production on a seasonal basis during the dry season in the salterns of the Vinh Tien Shrimp-Salt Cooperative, Vin Chau District, Soc Trang Province, in the Mekong Delta, Vietnam. These field culture systems produce large numbers of diapause embryos, two groups of which have been studied in the present article, referred to as VN-1 (collected on July 29, 1993) and VN-2 (collected on February 27, 1994). These embryos were packed in plastic bags containing small volumes of water from their collection ponds and eventually flown to Bodega Bay. Dates of receipt were September 7, 1993 (VN-1), and March 13, 1994 (VN-2). In both cases the embryos were first incubated aerobically

TABLE 1

Incorporation of $NaH^{14}CO_3$ by diapause and activated (dried and rehydrated) embryos produced in laboratory cultures

Age of Diapause Embryos (d)	Small Molecules (cpm · mg dry wt^{-1})	Nucleic Acids (cpm · mg dry wt^{-1})	Proteins (cpm · mg dry wt^{-1})
0–3:			
In diapause	4,300	244	388
Activated	3,600	230	350
Activated/in diapause	1	1	1
12:			
In diapause	3,400	23	45
Activated	3,100	280	426
Activated/in diapause	1	12	10

Note. Embryos were incubated for 4 h at 25°C in SW (125 mg wet wt · 4 mL^{-1}) containing $NaH^{14}CO_3$ at a level of 5 $\mu Ci \cdot mL^{-1}$. The ratios "activated/in diapause" are rounded to the nearest whole number.

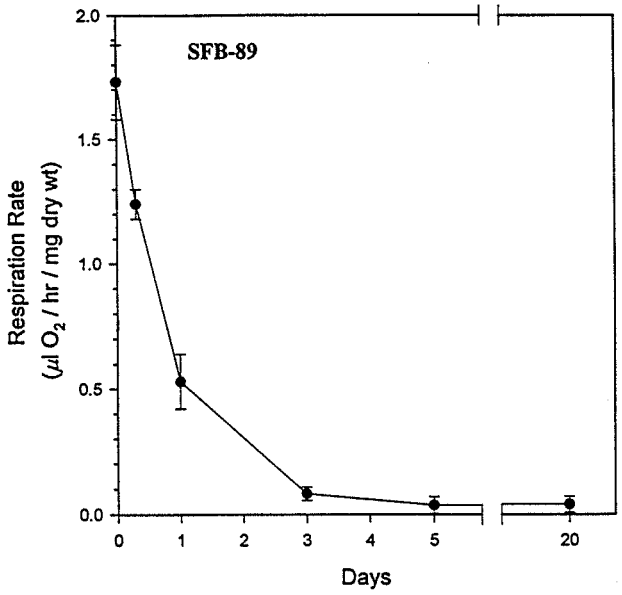

Fig. 1. Respiration rates of diapause embryos collected in the laboratory. The "0 time" data represent embryos collected within 24 h after release from maternal females. Solid circles represent means; bars represent ±1 standard error of the mean; n = *3.*

in the light for 4 d to allow embryos that had been activated (diapause terminated during shipment) to produce nauplius larvae (which were then discarded). The remaining embryos, still in diapause and floating on the surface, were stored in SW in large, covered petri dishes in the dark at 20°–24°C for the duration of this study.

Diapause Termination

Two methods were used: drying at relative humidity 55%–60% on filter paper discs for 24 h (light, 21°–24°C) followed by rehydration in SW; and aerobic incubation for 15 min in 0.4 M NaCl containing 3% H_2O_2. Both treatments are effective in terminating diapause in SFB embryos (Lavens and Sorgeloos 1987).

Hatching Assays

Embryos were placed into welled plastic depression plates, each of the 20 wells containing 10–30 embryos in 400 µL SW. The plates were covered, sealed

with tape, and incubated in constant light at 21°–24°C until hatching was deemed to be complete, usually after 5 d. Adequate molecular O_2 is present in these sealed plates, since controls (activated embryos) exhibited about 90% hatching levels within 2 d. Further details can be found in Clegg (1994). In all cases at least 200 embryos were used to determine hatching percentages.

Respiration Measurements

Embryos (5–50 mg wet wt) were placed in 2 mL of SW in the glass cell of a Strathkelvin recording O_2 electrode system at 25° ± 0.1°C. In some cases (fig. 1) 1 mL of 0.125 M Na phosphate buffer (pH 7.6) was used in the respiration cell. However, subsequent work showed that these conditions produced the same results as SW. The embryos were mixed at a constant rate with a small magnetic stir bar. Oxygen consumption was calculated from the decrease in % saturation (air) with time, expressed as $\mu L\ O_2 \cdot h^{-1} \cdot$ mg dry wt^{-1}. Complete details of these procedures and calculations have been published (Clegg 1993).

Incorporation of $NaH^{14}CO_3$

Although these embryos are known to be impermeable to nonvolatile solutes, $^{14}CO_2$ (from $H^{14}CO_3^-$) does penetrate and is incorporated into a variety of amino acids, other organic acids, and, under aerobic conditions, pyrimidine nucleotides (see Clegg 1976). In this way the synthesis of proteins and RNA can be measured in activated embryos under permissive conditions (see Clegg 1977). We should point out that DNA synthesis does not occur in encysted embryos until they emerge from their shells (reviewed in MacRae et al. [1989], Warner et al. [1989], and Browne et al. [1991]). Embryos (diapause and activated) were incubated for 4 h in SW or 0.4 M NaCl solutions buffered to pH 7.2 with 0.1 M phosphate buffer, containing $NaH^{14}CO_3$ (Amersham) at levels specified in tables 1 and 2. Following aerobic incubation at 25°C the embryos were thoroughly washed with ice-cold distilled water and homogenized in cold 5% trichloroacetic acid (TCA) (100 mg wet wt embryos $\cdot$ mL^{-1}) with glass grinders. The homogenate was fractionated as follows: the supernatant obtained from centrifugation of the homogenate at 2,000 *g* for 10 min (4°C) is referred to as "small molecules"; after the resulting pellet was washed in 200 times its volume of cold 5% TCA the pellet was resuspended in 1.0 mL 5% TCA and heated at 90°C for 1 h, cooled to 0°C, and centrifuged (200 *g*, 20 min at 4°C), the supernatant being referred to as "nucleic acids"; the resulting final pellet was washed in 200 times its volume of 5% TCA, resuspended in 1.0 mL 88% formic acid, and referred to as "proteins." Aliquots of

TABLE 2
Incorporation of $NaH^{14}CO_3$ by diapause and activated (by H_2O_2) embryos collected from the field (Vietnam)

Group and Age of Diapause Embryos	Small Molecules (cpm · mg dry wt^{-1})	Nucleic Acids (cpm · mg dry wt^{-1})	Proteins (cpm · mg dry wt^{-1})
VN-1, 69 d:			
In diapause	70,400	30	41
Activated	110,200	1,170	4,250
Activated/in diapause ...	2	39	104
VN-2, 30 d:			
In diapause	86,700	55	87
Activated	103,100	1,200	3,200
Activated/in diapause ...	1	22	37

Note. Embryos were incubated for 4 h at 25°C in 0.4 M NaCl buffered to pH 7.2 and 0.1 M phosphate buffer and containing 25 $\mu Ci \cdot mL^{-1}$ $NaH^{14}CO_3$. See Material and Methods for further details. The ratios "activated/in diapause" are rounded to the nearest whole number.

these fractions were assayed for ^{14}C in a liquid scintillation spectrometer and the results expressed on a dry weight basis.

In some cases, the embryos after incubation with $NaH^{14}CO_3$ were homogenized at 0°C in buffer K, which has been shown to be suitable for cell fractionation in this system (Clegg, Jackson, and Warner 1994): 150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, and 50 mM Hepes buffered to pH 7.2. Homogenates were centrifuged at 1,630 *g* for 5 min at 2°C to obtain supernatant (S) and pellet (P) fractions; the latter were washed once with 200 times their volume of buffer K and restored to their original volume prior to electrophoresis and autoradiography.

Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Aliquots of S and P were added to 2× sample buffer (Laemmli 1970), heated at 100°C for 5 min in capped microfuge tubes, and centrifuged (2,000 *g*, 5 min). These supernatants were electrophoresed by SDS-PAGE on 12% polyacrylamide gels, and proteins were detected by Coomassie blue-G. Following drying, the gels were exposed to x-ray film for autoradiography.

Thin-Layer Chromatography

Aliquots of the cold 5% TCA-soluble fraction were dried at the base of 100-μm-thick cellulose sheets (Eastman Kodak, Rochester, N.Y.), which were developed with 7 volumes isopropanol, 2 volumes water, and 1 volume 88% formic acid (ascending). After thorough drying the thin layers were exposed to x-ray film for autoradiography.

Results

Respiration of Recently Released Diapause Embryos (Laboratory Cultures)

Figure 1 records the respiration rates of diapause embryos that had been released from females over a 24-h period (0 time) and measured at periods thereafter. The initial very high rate was comparable to that of activated embryos (Clegg 1993) but fell to barely detectable levels after about 4 d. Some of the respiration at this time, and thereafter, is due to the presence of embryos that broke diapause (about 9%) and produced larvae during this 20-d period. If we assume that the embryos terminating diapause during the 5–20-d period (fig. 1) are respiring at the average "0 time" rate (1.73 $\mu L\ O_2 \cdot h^{-1} \cdot mg^{-1}$) and take the average percentage of embryos terminating diapause on any given day over this period (9%/15 = 0.6%), then the contribution of these activated embryos will be roughly 0.01 $\mu L\ O_2 \cdot h^{-1} \cdot mg^{-1}$. That estimated rate can be compared to the measured value of 0.04 $\pm$ 0.02 $\mu L\ O_2 \cdot h^{-1} \cdot mg^{-1}$ (fig. 1). Although this exercise is viewed only as a rough approximation, it does suggest that the embryos remaining in diapause are respiring at even lower rates, if at all, over the 5–20-d period (fig. 1).

When samples of the "0 time" and 20-d-old diapause embryos were air dried and then rehydrated in SW, about 90% produced larvae within 48 h. Therefore, the population of embryos used in this study was indeed in diapause in the sense that development was arrested. On the other hand, as we have shown (fig. 1), recently released embryos exhibited a vigorous respiratory metabolism. To examine the scope of this metabolism we studied the ability of diapause embryos to synthesize macromolecules.

Incorporation of $NaH^{14}CO_3$ by Diapause Embryos (Laboratory Cultures)

In this study (table 1) diapause embryos produced over a 3-d period were used. The sample was composed of embryos that had just been released or

that had been released sometime during the 3-d collection period (0–3 d). In the other case, a similar 0–3-d collection of embryos was kept for 2 wk prior to study. In both cases a sample of embryos was air dried and then rehydrated prior to study. As mentioned, drying is a treatment known to terminate diapause in these embryos.

Because of the difficulty of obtaining large numbers of diapause embryos from laboratory cultures this study was limited to a single large sample. Nevertheless, the results seem clear (table 1): recently released diapause embryos exhibited an incorporation into all fractions comparable to that of activated (air-dried) embryos. In contrast, diapause embryos that were studied about 2 wk after their release from females showed a marked reduction of incorporation into macromolecules (table 1). We delay comment on the nature of $^{14}CO_2$ incorporation into the small molecules fraction until the Discussion, only noting now that diapause embryos are also very active in that regard.

Few of these diapause embryos terminated diapause during the period of study: fewer than 1% in the 0–3-d sample and just over 4% during the 2-wk period. It is quite possible that some of the very low incorporation into macromolecules observed in the 2-wk population could be due to these activated embryos (table 1). In contrast, about 85% of these embryos produced larvae after drying and rehydration (both cases).

Studies on Diapause Embryos Collected from the Field

In this section we describe results from two samples of embryos produced in Vietnam, referred to as VN-1 and VN-2 (see Material and Methods).

Figure 2 describes the almost undetectable respiration rate exhibited by diapause embryos 67 d after their collection from the field. The effects of treatments that terminate diapause (drying and H_2O_2) are also shown. As expected from previous work (Clegg 1993), dried and rehydrated embryos exhibited an increasingly active respiration as metabolism and development resumed. In the case of H_2O_2 treatment a lag of about 1.5 h was evident, indicating the time required for this oxidant to somehow overcome the mechanism(s) that maintain diapause.

Comparable studies were carried out on a second independent sample from Vietnam (VN-2). Respiration measurements were performed as soon as the embryos were processed at Bodega Bay and after prolonged storage (fig. 3). Note first that the level of respiration exhibited by diapause embryos decreased with storage time, notably during the first few hours over which respiration was measured. It appears that the transfer of embryos to the stirred respiration cell might activate a small portion of them, at least in the

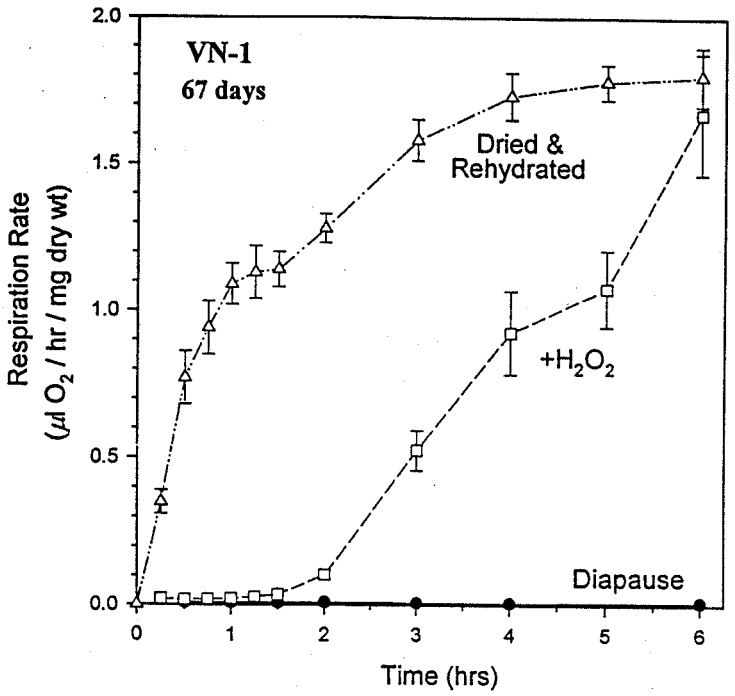

Fig. 2. Respiration rates of embryos produced in the field in Vietnam (VN-1) 67 d after collection. Circles, diapause embryos; triangles, embryos activated by drying and rehydration; squares, embryos activated by H_2O_2 treatment.

94-d sample. Another notable feature of these studies concerns the increase in the lag associated with H_2O_2-induced respiration and diapause termination (fig. 3). That result suggested that the depth of diapause increased as the duration of diapause lengthened.

The ability of VN embryos to incorporate $^{14}CO_2$ was studied (table 2). Compared to its incorporation into macromolecules by embryos activated by H_2O_2, the incorporation of ^{14}C into macromolecules by diapause embryos was greatly reduced and just measurable. As mentioned previously, the matter of incorporation into the small molecule fraction will be delayed until the Discussion. We point out that the data in table 2 should not be compared directly (quantitatively) to those obtained from embryos produced in laboratory cultures (table 1). That is so because the specific activity of $^{14}CO_2$ was much greater in the studies described in table 2 than in those described in table 1. Nevertheless, the combined results clearly indicate that the synthesis of macromolecules is greatly suppressed during prolonged diapause.

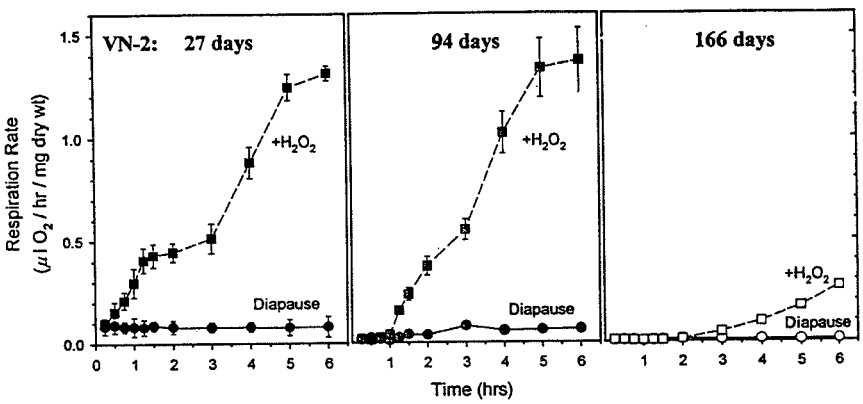

Fig. 3. Respiration rates of embryos from Vietnam (VN-2) measured at different times after collection from field cultures. Circles, diapause embryos; squares, embryos activated by H_2O_2 treatment prior to measurement.

Further study of the reduction in macromolecular synthesis in diapause embryos was carried out by SDS-PAGE and autoradiography (fig. 4) on the VN-1 sample (75 d after collection from the field). As described in the Material and Methods section, the embryos were fractionated into low-speed S and P after $^{14}CO_2$ incubation but prior to electrophoresis. Recall also that P is composed chiefly of nuclei and yolk platelets. Figure 4*A* shows protein profiles revealed by Coomassie staining. Those profiles were very similar for diapause (*D*) and H_2O_2-activated (*H*) embryos with one marked exception: substantial amounts of a major protein of about 26,000 molecular mass (*arrowheads*) appear to be translocated from the S to the P fraction in extracts of diapause embryos. In the Discussion section we will describe previous work on this abundant protein (referred to as p26), which indicates that a similar translocation also occurs in activated embryos exposed to various forms of stress.

An autoradiograph of this dried gel is shown in figure 4*B*. As expected, ^{14}C incorporation into the macromolecule fractions of diapause embryos was so low (table 2) that very little was observed in the autoradiograph (*Ds, Dp*). On the other hand, a wide variety of proteins was labeled in the H_2O_2-activated embryo (*Hs, Hp*), apparently including p26, which was present only in the supernatant fraction. Figure 4 is a representative of three separate studies that revealed comparable outcomes.

The possibility arose that diapause embryos failed to exhibit an appreciable synthesis of proteins because of the lack of a ^{14}C-labeled amino acid pool. To evaluate this option we examined the small molecule fraction from the

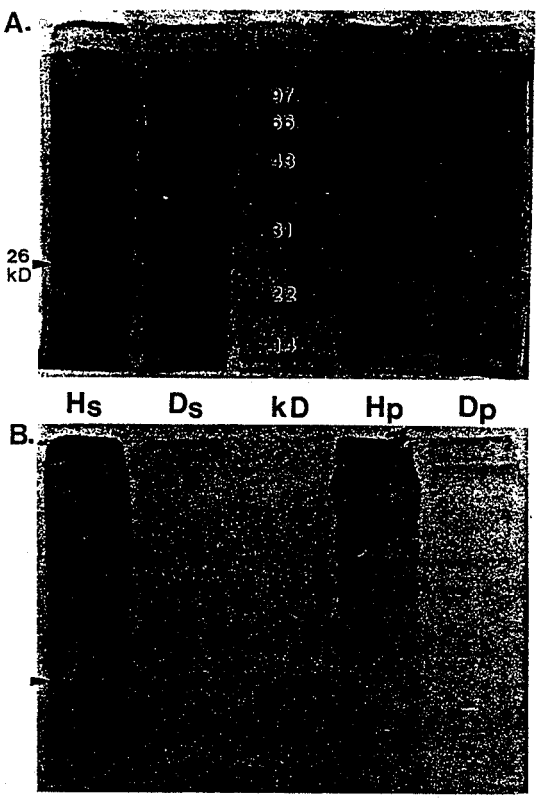

Fig. 4. Denaturing polyacrylamide gel electrophoresis of extracts (S and P) from diapause (D) and H_2O_2-activated (H) embryos from Vietnam after incubation with $NaH^{14}CO_3$. These embryos were from one of the populations used in the incorporation studies (table 2: VN-2, 30 d). Conditions of incubation are the same as in table 2. See Material and Methods for preparation of the low-speed S and P. Part A *shows gels stained with Coomassie blue-G and dried; part* B *represents the same gel after autoradiography (90 d exposure at 21°–23° C). Molecular mass standards (*kD*) are shown in part* A. *The arrowheads mark the location of a protein referred to in the text as p26. Aliquots equivalent to the same number of embryos were applied in all lanes.*

same population of embryos used for the study shown in figure 4. Thin-layer chromatography-autoradiography (fig. 5) showed that at least three amino acids in diapause embryos were labeled with ^{14}C. Although there are obvious differences between the patterns of ^{14}C-labeling of small molecules by activated and diapause embryos, this result showed that the lack of in-

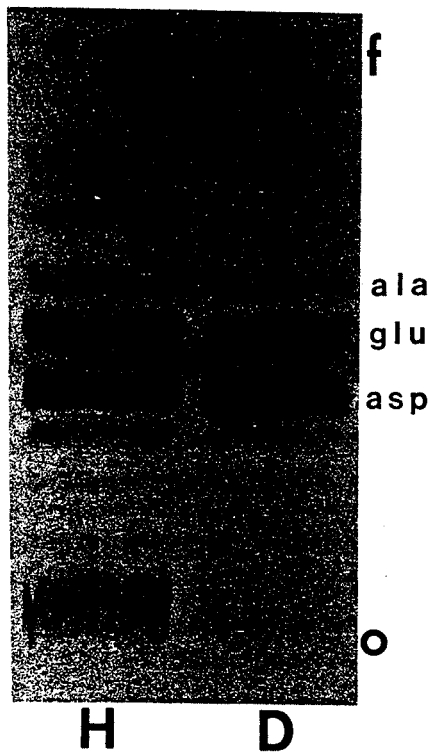

Fig. 5. Thin-layer chromatography (autoradiogram) of the small molecule fraction from H_2O_2-activated (H) *and diapause* (D) *embryos. These embryos were from the same sample described in table 2 and fig. 4 (VN-2, 30 d after collection). Incubation conditions were also the same (see table 2). The application origin is marked by* o *and the solvent front by* f. *Locations are given for alanine* (ala), *glutamic acid* (glu), *and aspartic acid* (asp), *which have been identified in previous work (Clegg 1976, 1977).*

corporation of ^{14}C into protein was not due to the absence of a labeled amino acid pool.

Discussion

Brief comment on the term "diapause" is in order. While diapause is always associated with a cessation of development in embryos and growth in other life history stages, the extent to which a reduction in metabolic rate occurs varies widely depending on the particular organism (Lees 1955; Clutter

1978; Drinkwater and Clegg 1991). The studies presented here have used a single species, *Artemia franciscana,* originating from the San Francisco Bay area. However, we should note again that the work was carried out on separate samples, obtained over a period of years, as well as on those produced in Vietnam. No marked differences between these different origins were noted with regard to the parameters studied here. Therefore, we believe the conclusions to be drawn are valid for the SFB population in general.

Somewhat unexpected was the existence of an active metabolism in diapause embryos recently released from females (fig. 1). As mentioned, metabolic depression is only one of the criteria used to characterize the state of diapause, and it is only one of the criteria applied to *Artemia* spp. (Lavens and Sorgeloos 1987; Drinkwater and Clegg 1991). That criterion also applies to diapause embryos of *A. franciscana,* but only after several days following their release into the environment. Thus, embryonic diapause is separated from metabolic rate depression. From those observations we conclude that the regulatory mechanisms causing metabolic depression are most likely due to the intrinsic properties of the cells of the diapause embryo, and not the direct result of maternal influence. It also appears that the mechanisms that arrest embryonic development are not identical to those involved with metabolic rate depression. That conclusion is drawn because development has already been arrested in the embryo at, or even before, its release from the maternal female.

Another feature of this system involves the depth of diapause, which appears to increase as the duration of diapause continues. For example, the rate of diapause embryo respiration decreases (fig. 1), and the time required for termination of diapause by treatment with H_2O_2 is extended (fig. 3), both as the duration of diapause is prolonged. Thus, the ability of a given embryo to respond to diapause-terminating conditions may be more a function of the previous duration of its diapause than its inherent "viability." In other words, it is possible that these embryos will eventually find themselves locked irreversibly in diapause.

Although treatment with H_2O_2 has been shown to be a very effective way to terminate diapause in SFB *Artemia* (reviewed by Lavens and Sorgeloos [1987]), its mode of action is unknown. One may suppose that an understanding of the latter would provide insight into the mechanism(s) that maintain diapause. It is worth noting that H_2O_2 causes profound effects on a variety of cells at moderate micromolar concentrations (see Schubert and Wilmer 1991; Martins and Meneghini 1994). However, this compound must be present in high millimolar concentrations before it is effective as a terminator of diapause in *A. franciscana.* For example, 3% H_2O_2 was used in the present study, equivalent to 830 mM. It is remarkable that these embryos

survive immersion in such high H_2O_2 concentrations let alone for them to utilize it in the process of diapause termination (table 2, figs. 2 and 3). Thus, it is possible that H_2O_2 does not enter the embryonic compartment or that only small amounts are allowed to cross the outer shell. Further study of the mode of action of H_2O_2 seems worthwhile.

Evaluation of metabolism in *Artemia* spp. embryos is seriously hampered by their impermeability to appropriate radioactive precursors, and we have had to resort to the use of $^{14}CO_2$ incorporation (Clegg 1976, 1977). The pathways of CO_2 fixation have not been worked out in this system but would appear to be similar to those in other heterotrophs, involving several carboxylation reactions (Patel 1989) followed by transamination to obtain a variety of amino acids (see Biggers and Belvé 1974). Although incorporation of $^{14}CO_2$ into the small molecule fraction by diapause embryos is somewhat less than incorporation by activated ones it is, nevertheless, substantial over the 4-h period of measurement (table 2; fig. 5). Does this incorporation reflect an ongoing metabolism in embryos undergoing prolonged diapause? If this incorporation involves a net fixation of $^{14}CO_2$, then it is clear that free energy (ATP or other ribonucleoside triphosphates) must be provided: keq for net carboxylation is in the range 10^{-5}–10^{-6} M, corresponding to standard free energy requirements ($\Delta G°$) of 29–34 kJ · mol^{-1} (Fasman 1976, p. 303). The alternative is that $^{14}CO_2$ simply equilibrates enzymatically with the appropriate carboxyl group(s) of metabolites (such as oxaloacetate), without the need for free energy input or a change in their concentration(s). Although net carboxylation reactions are commonly referred to as "irreversible," that does not apply to the isotopic labeling being discussed here. A similar line of reasoning applies to transamination reactions, which are fully reversible and would label appropriate carboxyl groups of certain amino acids, including those shown in figure 5. In view of the evidence that the metabolism of embryos in prolonged diapause is severely reduced if it occurs at all (figs. 1 and 4; table 2) we believe the isotopic labeling interpretation is much more likely than the net fixation of $^{14}CO_2$.

The ^{14}C-labeled amino acid pool in diapause embryos (fig. 5) is adequate to reveal protein synthesis. Therefore, we conclude that protein synthesis in the diapause embryo must be severely depressed as diapause is prolonged. Furthermore, the very low incorporation that we do observe could be due to the small number of embryos that are activated during the study, and perhaps to contributions from the microbial flora associated with the shells of these embryos (Wheeler, Yudin, and Clark 1979; Coleman et al. 1980). Finally, we note that the fraction referred to as "protein" (tables 1 and 2) is not highly purified protein and could contain small amounts of ^{14}C-labeled compounds other than proteins. Consequently, it seems that the diapause

embryo exhibits remarkable stability in the virtual absence of an ability to synthesize proteins (and probably anything else), and without an ongoing energy metabolism. We see no evidence of proteolysis in diapause embryos (fig. 4*A*) and can only assume that pathways of macromolecular degradation are similarly brought to a standstill during diapause. Thus, as of this writing (November 1994) the VN-2 embryos used in this study have been in continuous diapause for about 9 mo. Yet about 85% produced normal larvae when diapause was terminated by H_2O_2. While one can envision how hydrolase activities can be controlled under these conditions, it is more difficult to understand how the thermal unfolding and subsequent aggregation of proteins is avoided. This is so because globular proteins, in general, are quite unstable at physiological temperatures (Dill 1990; Doig and Williams 1992). One possibility involves the presence in these embryos of large amounts of trehalose and glycerol (Dutrieu 1960; Clegg 1962), both being compatible solutes that might stabilize proteins against denaturation (see Carpenter et al. 1993). Another possibility that we take up next involves the potential participation of molecular chaperones to prevent unfolding and aggregation (for recent reviews see Georgopolous and Welch [1993], Hendrick and Hartl [1993], and Ellis [1994]).

A major difference between the proteins of activated and diapause embryos as revealed by SDS-PAGE concerns a protein of about 26,000 molecular mass, p26 (fig. 4*A*). In activated embryo extracts virtually all of this protein is located in the low-speed supernatant, whereas it is roughly equally divided between the two fractions (S and P) from diapause embryos. That result is particularly interesting, since previous studies on p26 show that it exhibits a similar distribution in activated embryos exposed to anoxia and thermal shock (Clegg et al. 1994). That study also showed that p26 in the low-speed pellet is associated solely with the nuclei of anoxic embryos, makes up about 15% of the total nonyolk protein, is found only in the encysted embryo stage of the life cycle, and undergoes translocations to other intracellular locations during anoxia (i.e., within the low-speed supernatant). In contrast, p26 in dried embryos hydrated at 0°C, and in those incubated aerobically at 25°C, exists as a "soluble" aggregate of about 500,000 molecular mass (Clegg et al. 1994). Thus, it is possible that these intracellular translocations of p26 are part of the same system that stabilizes activated embryos during anoxia and thermal (heat and cold) shock, as well as those undergoing diapause (fig. 4). We previously proposed that p26 plays the role of a molecular chaperone to protect globular proteins against thermal unfolding during prolonged anoxia (Clegg et al. 1994). Perhaps p26 plays a similar role in the diapause embryo. That these embryos are indeed exceptionally stable has been shown by their ability to undergo 2 yr, at the least, of con-

tinuous anoxia (Clegg 1994), during which there is good evidence that metabolism comes to a reversible standstill (see Hand [1991] and particularly Hontario et al. [1993]). If the activated embryo can accomplish that remarkable feat during anoxia, we see no reason why the diapause embryo cannot.

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Literature Cited

BIGGERS, J. D., and A. R. BELVÉ. 1974. Carbon dioxide in developmental processes. Pages 87–105 in G. NAHAS and K. E. SCHAEFER, eds. Carbon dioxide and metabolic regulations. Springer, Berlin.

BROWNE, R. A., P. SORGELOOS, and C. N. A. TROTMAN, eds. 1991. The biology of *Artemia.* CRC, Boca Raton, Fla. 286 pp.

CARPENTER, J. F., J. S. CLEGG, J. H. CROWE, and G. N. SOMERO, eds. 1993. Compatible solutes and macromolecular stability. Cryobiology 30:201–241.

CLEGG, J. S. 1962. Free glycerol in dormant cysts of the brine shrimp *Artemia salina* and its disappearance during development. Biol. Bull. 123:295–301.

———. 1965. The origin of trehalose and its significance during the formation of encysted dormant embryos of *Artemia salina.* Comp. Biochem. Physiol. 14:135–143.

———. 1976. Interrelationships between water and cellular metabolism in *Artemia* cysts. V. $^{14}CO_2$-incorporation. J. Cell. Physiol. 89:369–380.

———. 1977. Interrelationships between water and cellular metabolism in *Artemia* cysts. VI. RNA and protein synthesis. J. Cell. Physiol. 91:143–154.

———. 1993. Aerobic metabolism of *Artemia franciscana* embryos after continuous anoxia over a one year period. J. Comp. Physiol. 163B:48–51.

———. 1994. The unusual response of *Artemia franciscana* embryos to anoxia. J. Exp. Zool. 270:332–334.

CLEGG, J. S., S. A. JACKSON, and A. H. WARNER. 1994. Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana.* Exp. Cell Res. 212:77–83.

CLUTTER, M. E., ed. 1978. Dormancy and developmental arrest. Academic Press, New York. 316 pp.

COLEMAN, D. E., L. K. NAKAGAWA, R. M. NAKAMURA, and E. CHANG. 1980. The effects of antibiotics on the hatching of *Artemia* cysts. Pages 153–157 in G. PERSOONE, P. SORGELOOS, O. ROELS, and E. JASPERS, eds. The brine shrimp *Artemia.* Vol. 3. Universa, Wetteren, Belgium.

DILL, K. A. 1990. Dominant forces in protein folding. Biochemistry 29:7133–7155.

DOIG, A. J., and D. H. WILLIAMS. 1992. Why water-soluble, compact globular proteins have similar specific enthalpies of unfolding at 110°C. Biochemistry 31:9371–9375.

DRINKWATER, L. E., and J. S. CLEGG. 1991. Experimental biology of cyst diapause. Pages 93–117 in R. A. BROWNE, P. SORGELOOS, and C. N. A. TROTMAN, eds. *Artemia* biology. CRC, Boca Raton, Fla.

DRINKWATER, L. E., and G. H. CROWE. 1987. Regulation of embryonic diapause in *Artemia:* environmental and physiological signals. J. Exp. Zool. 241:297–307.

DUTRIEU, J. 1960. Observations biochimiques et physiologiques sur le dévelopment d'*Artemia salina* Leach. Arch. Zool. Exp. Gen. 99:1–133.

ELLIS, R. J. 1994. Roles of molecular chaperones in protein folding. Curr. Opinion Structural Biol. 4:117–122.

FASMAN, G. D., ed. 1976. Physical and chemical data. Vol. 1 of Handbook of biochemistry and molecular biology. 3d ed. CRC, Boca Raton, Fla.

GEORGOPOLOUS, C., and W. T. WELCH. 1993. Role of major heat-shock proteins as molecular chaperones. Annu. Rev. Cell Biol. 9:601–634.

HAND, S. C. 1991. Metabolic dormancy in aquatic invertebrates. Pages 1–16 in R. GILLES, ed. Advances in comparative and environmental physiology. Vol. 8. Springer, Berlin.

HENDRICK, J. P., and F.-U. HARTL. 1993. Molecular chaperone functions of heat shock proteins. Annu. Rev. Biochem. 62:349–384.

HONTARIO, F., J. H. CROWE, L. M. CROWE, and F. AMAT. 1993. Metabolic heat production by *Artemia* embryos under anoxic conditions. J. Exp. Biol. 178:149–159.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685.

LAVENS, P., and P. SORGELOOS. 1987. The cryptobiotic state of *Artemia* cysts, its diapause deactivation and hatching: a review. Pages 27–63 in P. SORGELOOS, D. A. BENGSTON, W. DECLEIR, and E. JASPER, eds. *Artemia* research and its applications. Vol. 3. Universa, Wetteren, Belgium.

LEES, A. D. 1955. Physiology of diapause in arthropods. Cambridge University Press, Cambridge. 196 pp.

MACRAE, T. H., J. C. BAGSHAW, and A. H. WARNER, eds. 1989. Biochemistry and cell biology of *Artemia.* CRC, Boca Raton, Fla. 264 pp.

MARTINS, E. A. L., and R. MENEGHINI. 1994. Cellular DNA damage by hydrogen peroxide is attenuated by hypotonicity. Biochem. J. 299:137–140.

MCNAMARA, J. M. 1994. Timing of entry into diapause: optimal allocation to "growth" and "reproduction" in a stochastic environment. J. Theor. Biol. 168:201–209.

PATEL, M. S. 1989. CO_2-fixing enzymes. Pages 309–341 in A. A. BOULTON and G. B. BAKER, eds. Neuromethods. Vol. 11. Humana, Clifton, N.J.

SCHUBERT, J., and J. W. WILMER. 1991. Does hydrogen peroxide exist "free" in biological systems? Free Radic. Biol. Med. 11:545–555.

SORGELOOS, P., D. A. BENGSTON, W. DECLEIR, and E. JASPERS, eds. 1987. *Artemia* research and its applications. Vols. 1–3. Universa, Wetteren, Belgium. 1,498 pp.

VAN DER LINDEN, A., R. BLUST, A. J. VAN LAERE, and W. DECLEIR. 1988. Light-induced release of *Artemia* embryos from diapause: analysis of metabolic status. J. Exp. Zool. 247:131–140.

WARNER, A. H., T. H. MACRAE, and J. C. BAGSHAW, eds. 1989. Cell and molecular biology of *Artemia*. Plenum, New York. 453 pp.

WHEELER, R., A. I. YUDIN, and W. H. CLARK, JR. 1979. Hatching events in the cysts of *Artemia salina*. Aquaculture 18:59–67.

YAMASHITA, O., and K. HASEGAWA. 1985. Embryonic diapause. Pages 407–434 in G. A. KERKUT and L. I. GILBERT, eds. Comprehensive insect physiology, biochemistry and pharmacology. Vol. 1. Pergamon, New York.